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Letter to the Editor

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To the Editor,

The widespread use of immunoassays can be attributed to their analytical sensitivity combined with the possibility of direct measurement in unprocessed serum or plasma samples. However, a complex matrix such as serum can challenge specificity or result in analytical interference [1]. Apart from interference due to hemolysis or lipemia, interference can occur due to compounds with a similar structure as the analyte or through the occurrence of human antibodies with affinity for immunoassay components [2]. In the last case, assay setup and differences in assay sensitivity for interference can result in seemingly normal, falsely elevated or low analyte concentrations, resulting in the possibility of errors in diagnostic and therapeutical decisions. Identifying the origin and specificity of interference is in most cases not straightforward and often requires investment in laboratory resources. However, patient care will benefit when both the origin of interference and the extent to which the interference affects different assays is known.

We recently encountered a case in which interference in Roche hormone immunoassays suggested hyperthyroidism. A 40-year-old male visited his general practitioner with intestinal problems and joint pain. His general practitioner ordered thyroid-stimulating hormone (TSH) and free thyroxine (T4) analysis [free T4 29.8 pmol/L (reference interval 10–24 pmol/L); TSH 0.28 mU/L (reference interval 0.5–5 mU/L)]. Based on the biochemical parameters, antithyroid drug treatment was started (30 mg thiamazole daily). However, the biochemical manifestations of hyperthyroidism remained unchanged in the following month and the physical problems did not resolve on which the dosage of thiamazole was increased to 60 mg daily. As antithyroid drug treatment was still ineffective after 2 months, matrix interference in the TSH and free T4 immunoassays was suspected and thiamazole treatment was discontinued. Additional blood analysis showed an elevated cortisol concentration of 877 nmol/L (reference interval 150–700 nmol/L) and a testosterone concentration of 37.6 nmol/L (reference interval 10–30 nmol/L) both measured in a blood sample drawn at 8 a.m.

To gain further insight into the extent of interference, additional hormones were measured using Roche immunoassays characterized by differences in assay setup and labeling of tracer and antibody (Table 1). Comparison with alternative assays suggested interference in the Roche T3 and prolactin assays as well. We encountered complex results in the attempt to identify the origin of interference (Table 1). Firstly, a doubling dilutions test showed sustained elevated cortisol concentrations. For testosterone, doubling dilutions gave a sustained elevated concentration in three out of the four dilutions. Adding 1/5 volume of mouse serum to control human male serum resulted in an expected reduction in testosterone concentration of 20%. However, addition of mouse serum to our patient's serum unexpectedly resulted in an increase of the already elevated testosterone concentration by 13%. Based on these results, an immunological origin of interference was suspected. To corroborate our hypothesis, we used heat treatment of serum (20 min at 55 °C or 60 °C) to dissociate immune complexes and thereby increasing free antibody concentration and possibly interference. This resulted in an increase of the already falsely elevated cortisol concentration by 75%. The falsely elevated testosterone

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Table 1: Assay details and analysis of interference.

	Control serum	Patient serum					
		Free T4	T3	Cortisol	Testosterone	TSH	Prolactin
Assay details and measurements							
Roche assay setup	n/a	Competitive	Competitive	Competitive	Competitive	Sandwich	Sandwich
Biotin label component	n/a	Tracer	Tracer	Antibody	Antibody	Antibody	Antibody
Ruthenium label component	n/a	Antibody	Antibody	Tracer	Tracer	Antibody	Antibody
Concentration Roche assay	n/a	29.8 pmol/L	2.7 nmol/L	877 nmol/L	37.6 nmol/L	0.28 mU/L	116 mU/L
Reference interval	n/a	10–24 pmol/L	1.1–3 nmol/L	150–700 nmol/L	10–30 nmol/L	0.4–4 mU/L	<350 mU/L
Alternative assay	n/a	Delfia, Wallac, fluoro-immunoassays	In house, radioimmunoassay	Routine LC tandem MS assay	Routine LC tandem MS assay	Delfia, Wallac, fluoroimmunoassay	Delfia, Wallac, fluoroimmunoassay
Concentration alternative assay	n/a	13.6 pmol/L	2.15 nmol/L	546 nmol/L	10.3 nmol/L	1.46 mU/L	201 mU/L
Reference interval	n/a	10–23 pmol/L	1.3–2.7 nmol/L	220–650 nmol/L	9–30 nmol/L	0.4–4 mU/L	<320 mU/L
Analysis of interference							
Doubling dilutions test	Linear (cortisol, testosterone)	n/d	n/d	Linear	Non-linear for 1:2 dilution	n/d	n/d
Effect mouse serum	No effect (testosterone)	n/d	n/d	n/d	Increased interference	n/d	n/d
Effect heat treatment	No effect (cortisol, testosterone)	n/d	n/d	Increased interference	Increased interference	n/d	n/d
Effect anti-human IgM	No effect (free T4, T3, cortisol, testosterone, TSH, prolactin)	Increased interference	Increased interference	Reduced interference	Reduced interference	Increased interference	Increased interference
Effect PEG precipitation	No effect (cortisol)	n/d	n/d	Reduced interference	n/d	n/d	n/d
Effect streptavidin	No effect (testosterone)	n/d	n/d	n/d	no effect	n/d	n/d
Effect $RuCl_2$	No effect (testosterone)	n/d	n/d	n/d	no effect	n/d	n/d
Effect ruthenium label	No effect (testosterone)	n/d	n/d	n/d	no effect	n/d	n/d
Effect biotin	Value above measuring range (testosterone)	n/d	n/d	n/d	Below LLOD	n/d	n/d

n/a, not appilicable; n/d, not determined; LLOD, lower limit of detection.

concentration increased with an additional 50%. Cortisol and testosterone molecules were heat-stable at the indicated temperatures and incubation time. Using polyethylene glycol (PEG) precipitation [3] we tried to remove the interfering antibodies and immune complexes from solution. Spiking of samples with serum positive for thyroperoxidase antibodies was used as an internal control. With increasing PEG concentrations the recovery of cortisol in

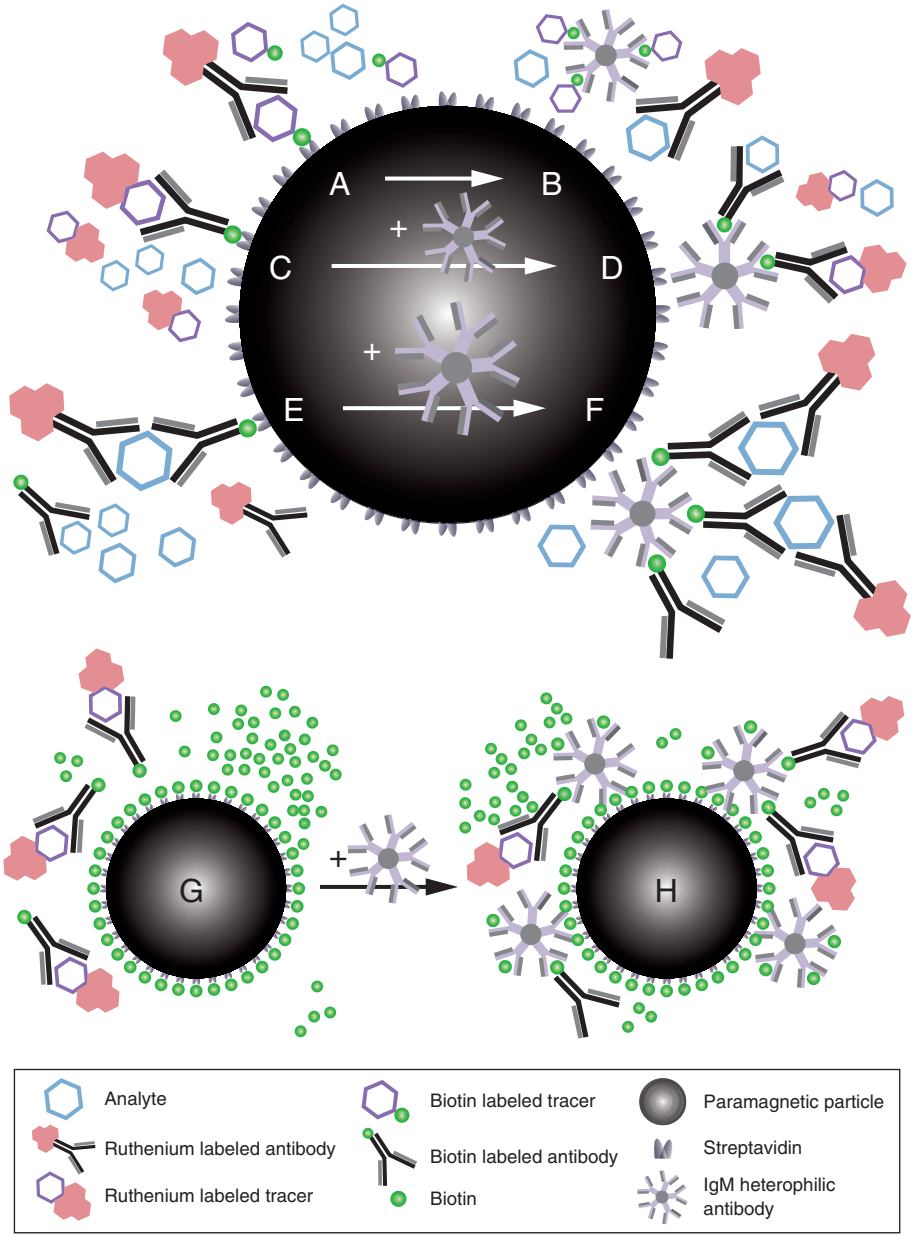

Figure 1: Assay setup and proposed method of interference.
(A) Competitive assay setup with biotin labeled tracer. Amount of binding of tracer (in complex with ruthenium labeled antibody) to paramagnetic bead is inversely proportional to the analyte concentration. (B) Interaction of heterophilic antibody with biotin labeled tracer prevents binding to paramagnetic bead. (C) Competitive assay setup with biotin labeled antibody. (D) Interaction of heterophilic antibody with biotin labeled antibody prevents binding to paramagnetic bead. (E) Sandwich assay setup. (F) Interaction of heterophilic antibody with biotin labeled antibody-analyte-ruthenium complex prevents binding to paramagnetic bead. (G) Incubation of control serum with excess biotin results in binding of streptavidin on paramagnetic beads resulting in falsely elevated analyte concentrations. (H) Incubation of patient serum with excess biotin results in binding of streptavidin on paramagnetic beads. The heterophilic antibody forms a bridge between the paramagnetic bead bound biotin and biotin labeled tracer or antibody. This results in falsely lowered analyte concentrations.

the pellet fraction of our patient increased to 75%–80%, which suggested that the interference was originating from large proteins or protein complexes. No cortisol could be recovered from the pellet fraction of control serum.

As interfering antibodies are mainly IgM class antibodies [4], we incubated serum with antibodies directed against human IgM in an attempt to modulate the interference. This reduced the interference in the cortisol and testosterone assays but increased the interference in the free T4, T3, TSH and prolactin assays. This suggested that the interfering antibody might interact with assay components other than assay antibodies. As such, the interfering antibody would classify as a heterophilic antibody rather than a human anti-animal antibody. To clarify the specificity of the heterophilic IgM antibody we incubated serum from our patient with separate components of the Roche immunoassays for which interference by human antibodies has been demonstrated before [5–7]. The competitive assays used are characterized by either a tracer (Figure 1A) or analyte specific antibody (Figure 1C) labeled with biotin, which allows binding to streptavidin-coated magnetic microparticles. Indirect analyte quantification is obtained through chemiluminescent emission from a ruthenium complex linked to either a tracer or analyte specific antibody. The sandwich methods are characterized by two assay specific antibodies labeled with either biotin or a ruthenium label (Figure 1E).

No effect on interference was noticed when serum was incubated with agarose-streptavidin resin, ruthenium chloride or the ruthenium label. Finally, we analyzed if the interference could originate from antibodies interacting with biotin. Addition of biotin (final concentration 70 μg/mL) resulted in an expected testosterone concentration above the measuring range in control male serum. Interestingly, addition of biotin to patient serum resulted in a testosterone concentration below the limit of detection. This suggests the presence of a heterophilic IgM antibody which interacts with biotin. In competitive assays this would result in binding of the biotin labeled tracer (Figure 1B) or biotin labeled assay antibody (Figure 1D) resulting in elevated analyte concentrations. For the sandwich assay, interaction with the biotin labeled assay antibody would result in lowered analyte concentrations (Figure 1F). Addition of free biotin in the absence of interfering antibody blocks streptavidin binding to biotinylated assay components resulting in elevated analyte concentrations for competitive assays (Figure 1G) while in the presence of interfering antibody a surplus of ruthenium labeled components can be bound and affect assay results in the opposite direction (Figure 1H).

Biotin supplements have previously been reported as a cause of low parathyroid hormone concentration as increased free serum biotin mimics the biotinylated antibody used in the detection process of the assay [8]. Also, intake of large biotin doses has been the cause of misdiagnosis of Graves' disease [9]. As incubation of serum from our patient with agarose-streptavidin resin did not normalize the test results, interference due to elevated free biotin concentrations can be excluded. Therefore we conclude that the heterophilic antibody interacts with biotinylated assay components, resulting in varying analytical effects. Anti-biotin IgM antibodies were shown previously to occur in 3% of adults [10]. As biotinylation is widely applied in biochemical assays this could result in falsely lowered or increased analyte concentrations in a substantial amount of patients worldwide. Whether or not the interference will result in significant changes from the true analyte concentration depends on both antibody affinity for biotin and antibody concentration as well as on assay sensitivity for interference.

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